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Biological Sample Preparation and ^{41}Ca AMS Measurement at LLNL

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Abstract

Calcium metabolism in biology may be better understood by the use of ^{41}Ca labels, although detection by accelerator mass spectrometry (AMS) is required. Methodologies for preparation of urine samples and subsequent AMS measurement were investigated. Novel attempts at preparing CaH_2 were unsuccessful, but CaF_2 of sufficient purity could be produced by precipitation of calcium from urine as oxalate, followed by separation of calcium by cation exchange chromatography and washing the CaF_2 precipitate. The presence of some remaining impurities could be compensated for by selecting the appropriate accelerated ion charge state for AMS. The use of projectile x rays for isobar discrimination was explored as an alternative to the conventional dE/dx device.

Introduction

The biological metabolism of calcium enriched or made deficient in a particular isotope can be followed by the subsequent measurement of that isotope in pertinent samples. The first labels to be used were the relatively short lived radioisotopes that can be detected by their decay. Then, with the development of conventional mass spectrometry, stable isotopes were employed also, as the lack of radiation dose to the subject permitted experiments otherwise precluded. Now that accelerator mass spectrometry (AMS) can detect ^{41}Ca at natural levels, research with calcium isotopes might be further extended by the addition of this radioisotope, a label again markedly different from those used previously, to the cannon of tracers.

The use of ^{41}Ca is particularly attractive: the radiocalcium is so long lived ($t_{1/2} \approx 10^5$ yr) with the release of soft x rays upon decay, so rare (natural Ca is only 10^{-15} ^{41}Ca) and in principle plentiful (produced by inexpensive neutron activation of the major calcium isotope) that

^{41}Ca can be employed as a tracer when the other isotopes are precluded for physiological, radiological or economic reasons. In practice, the isotope's properties lend it to the use for the *in vivo* study of human Ca metabolism and especially bone calcium kinetics which can be particularly intractable to conventional experimentation.

The power of ultrasensitive AMS has recently begun to be applied to biomedical problems [1]. Another collaboration has already experimented with ^{41}Ca [2]. They have essentially marked a subject's bone for life with the radiocalcium to permit a long term study of bone resorption. AMS measurement of calcium recovered from the subject's urine to date show fluctuations in resorption possibly related to menstrual cycle. In the future, the effects of varying diet or otherwise challenging the subject may be examined. We are attempting a somewhat similar experiment except that the radiocalcium is continuously administered in the daily diet for an extended period instead of given as a single bolus. This permits the body's extra cellular fluid and readily exchangeable bone to achieve various steady state ^{41}Ca concentrations. By measuring the fluid plateau levels as reflected in the subject's urine ^{41}Ca concentration, in combination with more conventional measurements, it may be possible to derive a host of human calcium kinetics parameters relatively inexpensively. At present, at best, such parameters are inferred by confining the subjects to a metabolic ward and making balance studies of calcium consumed and excreted. As the AMS based technique potentially affords the use of significantly more subjects, human calcium kinetics may be more completely studied, including surveys with respect for different medical conditions, race, gender, age, etc..

This methodology paper describes our on-going research into the actual biological sample ^{41}Ca measurement. This includes aspects of sample preparation and the AMS measurement: we have attempted to prepare calcium obtained from human urine as the hydride or fluoride; we have investigated the uses of our conventional differential ion energy loss rate gas ionization detector and a novel detection system characterizing ions by x ray emission as they are brought to rest to separate isobars at the end of our spectrometer.

Sample preparation

The electron affinity of calcium is so small (0.043 eV) that sputter ion sources cannot produce the intense beams of negative ions required for AMS. Instead, CaH_2 and CaF_2 compound targets are bombarded to produce CaH_3^- and CaF_3^- respectively. Fortunately potassium does not form stable equivalent ions and so the interfering isobar ^{41}K is largely discriminated against in the ion source. The advantages of utilizing the hydride are that CaH_3^- is a more copious secondary ion than is CaF_3^-, that the efficiency of being scattered into desirably charged positive ions at the terminal of a 10 MV FN class tandem is greater for accelerated CaH_3^- and that the AMS backgrounds of as little as about 10^{-16} ^{40}Ca are lower. The latter are consequences of the smaller nature of the CaF_3^- ion: more energy is available to the calcium atom at the terminal; more ultimate interferences can be injected as compound ions at mass 98 than can at mass 44. However, the preparation of the hydride has to date involved the reduction of the calcium oxide precursor to calcium metal, followed by its conversion to the hydride, requiring technology and techniques not common to biochemistry laboratories [3]. Also, once prepared, the very hydroscopic hydride must be kept dry.

In keeping with the philosophy at the Center for AMS of encouraging collaborators to present prepared samples for measurement we have studied means of producing the hydride via the metal that might be more widely applicable. However, our attempts by novel procedures were unsuccessful. Either the reactions were incomplete or else the CaH_2 produced could not be separated in pure form.

Microscale (10 mg Ca) syntheses of calcium hydride were attempted in a dry (nitrogen) box or a tube furnace in anhydrous solvents and/or under anhydrous gases according to these reaction schemes:

1. $2CaX_2 + LiAlH_4 \Rightarrow 2CaH_2 + LiX + AlX_3$ where X = Cl or Br and solvent = diethyl ether or tetrahydrofuran (THF);
2. $CaX_2 + 2LiH \Rightarrow CaH_2 + 2LiX$ where X and solvent are same as in scheme 1;
3. $CaCl_2 + 2M + H_2 \Rightarrow CaH_2 + 2MCl$ where M = Na or Li and temperature = 800 °C;
4. $CaCl_2 + Zn + H_2 \Rightarrow CaH_2 + ZnCl_2$ where temperature = 800 °C;
5. $3CaCl_2 + 8NH_3 \Rightarrow 3Ca + 6NH_4Cl + N_2$, $3Ca + 3H_2 \Rightarrow 3CaH_2$ where temperatures for the first and second reactions = 800 and 400 °C.

The calcium halides were prepared from $CaCO_3$ and the corresponding hydrohalic acid, and dried before use. For scheme one, calcium was best solubilized as the bromide in THF. Reactions at 30 and 80 °C were incomplete, and separation of product from unreacted calcium halide was not possible. For scheme two, the solubility of LiH was so low that no reaction was observed even after refluxing at 30 °C for so long that the solvent escaped. Use of scheme three in a tube furnace was complicated by the difficulty of using small quantities of sodium and lithium sufficiently free of oxygen and hydrogen, even when highly pure lithium under argon was purchased and the tube furnace was loaded in the dry box. For scheme four there was evidence of reaction, but the molten $CaCl_2$ was corrosive to the silica and porcelain combustion boats that were used. Scheme five appeared to be the most promising for preparation of pure CaH_2. Treatment of molten $CaCl_2$ with anhydrous NH_3 resulted in evidence of formation of metallic calcium, but, again, corrosion of vessels by molten $CaCl_2$ was problematic.

With the failure to produce the hydride, we endeavored to identify the necessary procedures for a fluoride production method that might potentially be widely employed.

The sample material used was a complete 24-h urine collection from a normal, healthy, male adult. This was acidified to $pH<1.9$ by adding HCl (12 M) to dissolve calcium salts, and aliquots containing between two and ten mg Ca, as determined by atomic absorption spectrophotometry, were used in subsequent experiments (one mg of calcium is sufficient for an AMS measurement; smaller samples can be triturated with known amounts of ^{40}Ca). Some urinary aliquots of known calcium content were "spiked" with known volumes of spiking solution to provide "enriched" urinary calcium with 41/40 ratios between 10^{-11} and 10^{-9}. The added solution consisted of ^{41}C-enriched carbonate (neutron irradiated $^{40}CaCO_3$) reacted with HCl to form the chloride and made-up to volume with water. All chemicals were reagent grade. All labware was polypropylene.

Calcium from each urine sample was precipitated as the oxalate according to principles described previously [4]. Refer to table 1 for details. When decomposition of organic matter was desired, the oxalate precipitate was suspended in one ml of concentrated (16 M) HNO_3 and was transferred to microwave digestion vessel with one ml of concentrated HNO_3 used for washing. As an option, calcium was separated from other anions and cations by cation exchange chromatography using various resins and eluents. The ion exchange procedures were modified from the literature [5-7], and the procedure found to be most effective for maximal recovery of calcium with minimal carryover of sodium, potassium, magnesium, sulfur and phosphorus is shown in table 1. "Mock" urine solutions were prepared with Ca, Mg, Na, K, S, Cl and P as a mixture of salts dissolved in 0.08 M HNO_3 for determination of elution profiles from ion exchange resins with various solvents. Eluate fractions were analyzed for Na, K, Mg, and Ca by atomic absorption spectrophotometry. Recovery of calcium in eluted fractions was also determined by atomic absorption spectrophotometry; aliquots of calcium standard solution of known volume and calcium concentration were washed onto and eluted from columns with various solvents.

Finally, calcium from all sources (urines, mock urines, standards) was precipitated as CaF_2 and washed.

AMS measurements of standards and spiked samples prepared by oxalate precipitation, digestion, and CaF_2 precipitation the spiked samples and standards were as expected and the observed isotope ratio for unspiked standard calcium was 5×10^{-13} but that for unspiked urine calcium was close to 10^{-12}, due primarily to phosphorus interference. It was concluded that urine samples contain so much sulfur, phosphorus, sodium, magnesium, and potassium that calcium fluoride pure enough for analysis by AMS can not be obtained solely by gravimetric and decompositional procedures.

Ion exchange procedures were developed to isolate the calcium. Initially, hydrobromic acid (8 M) and perchloric acid (1.25 M) were tried as eluents to remove sodium, potassium, and magnesium from cation exchange columns before elution of calcium, unaccompanied by phosphate or sulfate, with 1.25 M HNO_3 [5,6]. With these elution schemes, separations were insufficiently complete: potassium was found in some calcium-containing fractions. Calcium recoveries were low if only fractions without potassium were used. Subsequently, it was found that the interfering ions could be effectively eluted with 0.08 M HNO_3, and the calcium could be recovered in good yield by using 4 M HNO_3 [7]. Sulfonated polystyrene resins are stable at room temperature in nitric acid up to a concentration of 7 M [8].

The second batch of samples (urines, mock urines, and standards) was prepared by oxalate precipitation, digestion, cation exchange chromatography, and CaF_2 precipitation. The variables in preparation were number of washes and length of drying of the CaF_2 precipitate. As shown by the AMS data, phosphorus interference was no longer a problem, and potassium content of samples was lower also. Observed ratios for unspiked samples were in the 4×10^{-13} range, similar to observed ratios for unspiked calcium standards. The more thorough the washing and drying, the higher were the beam currents and precision of measurement.

The third lot of samples was prepared by oxalate precipitation, cation exchange chromatography, and CaF_2 precipitation, i.e., digestion was omitted. Precipitates were washed twice and dried for 20 h at 100 °C in a vacuum oven (the low temperature is convenient when precipitates are dried, stored and shipped in polypropylene microcentrifuge tubes with o-ringed screw caps). Results were essentially the same as for the most thoroughly washed and dried samples in the second batch. The recoveries of calcium from the oxalate precipitation step were essentially quantitative. Recoveries of calcium from the ion exchange separations were between 80 and 90%. Recoveries of calcium from the fluoride precipitation step also were between 80 and 90%.

In summary: oxalate precipitation is convenient for separation of urinary calcium from most of the sodium, potassium, magnesium, phosphorus and sulfur, but the oxalate precipitates are not purely calcium; digestion of calcium oxalate can be omitted from the procedure when cation exchange chromatography is included; cation exchange chromatography is useful to separate calcium from residual potassium and to replace phosphate, sulfate, oxalate and chloride from urine with nitrate from the eluent; CaF_2 precipitates should be washed at least twice and dried thoroughly for best results.

AMS measurement

For AMS measurement the bulk solid CaF_2 was powdered, mixed with a similar amount of silver powder for improved thermal conductivity and pressed into our standard sample holders. Up to 64 samples were mounted in our source and CaF_3^- beams of 750 nA extracted and injected into the tandem. Doubling the amount of Ag per sample increased the current although it might then become too large for steady tandem operation. Further increasing the Ag fraction tended to reduce the CaF_3^- current.

Accelerated calcium ions were usually analyzed in the 8+ charge state with the terminal at 9 MeV. The stripping efficiency was about 3.5%, i.e. the radiocalcium detections were normalized to $^{40}Ca^{8+}$ currents of up to 220 nA. $^{41}Ca^{8+}$ ions were detected after passage through the high-energy spectrometer consisting of two magnets and a Wien filter. The measured absolute ^{41}Ca concentration of standards were typically only 0.7 of the theoretical values, presumably because of terminal fractionation. Various interferences (some from the material of the sample holders) also impinged the final detector including $^{46}Ti^{9+}$, $^{51}V^{10+}$, $^{41}K^{8+}$, $^{36}S^{7+}$ and $^{31}P^{6+}$ which could be usually sufficiently discriminated against with our gas ionization detector provided that pile-up effects were not significant [9]. An alternative scheme was investigated in case that should be so, however. At our accelerator energies the ion charge spectrum peaks at 7+ and twice the signal could be obtained in the detector by analyzing this state. However, although this approach removed the P and S interferences, but added $^{35}Cl^{6+}$ and other Ti signatures, it could be a problematic measurement. Ultimately similar backgrounds could be obtained by measuring either $^{41}Ca^{7+}$ or $^{41}Ca^{8+}$, but the latter was significantly more tolerant of terminal instabilities and spectrometer settings: the 8+ background is due to the scattered tail of nominally well separated $^{46}\mathrm{Ca}^{9+}$ and a smaller but less well differentiated $^{40}\mathrm{C}^{8+}$ signal; the detector is barely able to separate $^{41}\mathrm{Ca}^{7+}$ from $^{40}\mathrm{Ca}^{7+}$ and $^{42}\mathrm{Ca}^{7+}$ ions, having only slightly longer and shorter ranges in the detector gas, in addition to the ^{46}Ca presence. In all cases large angle scattering events in the detector gas were rejected by timing the interval between the pulses at the detector anodes.

We further explored our ^{41}Ca AMS capability by replacing the dE/dx detector with an alternative technology for the detection and identification of ion beams under developed at LLNL, which we are referring to as Projectile X ray AMS (PXAMS) [10]. Incident ions are identified by the characteristic x rays they emit when stopping in a thick target. For the detection of heavier ions, or ions at lower energies, PXAMS will allow cleaner separation and rejection of isobars than is possible with ionization detectors. We appreciated that PXAMS would not reject the Ca interferences, but would those of P and S, etc., as well as being more robust to accelerator instabilities. Potentially, PXAMS would permit ^{41}Ca measurement using smaller accelerators systems which cannot otherwise discriminate the stable isobar ^{41}K.

Following analysis in the AMS spectrometer set to transmit $^{41}\mathrm{Ca}^{8+}$ ions, the ions were incident on a $1.2\,\mathrm{mg}/\mathrm{cm}^2$ V foil. Induced x rays were detected by a $100\,\mathrm{mm}^2$ high resolution, high purity germanium (HPGe) detector placed directly behind the foil. The total solid angle subtended by the detector was approximately 3 sr. A $125\mu\mathrm{m}$ Be foil was placed between the V foil and the detector to stop the ions and to attenuate low energy x rays. We recorded x ray spectra for a series of ^{41}Ca standards for which the $^{41}\mathrm{Ca}/^{40}\mathrm{Ca}$ ratios ranged from 5×10^{-9} to 5×10^{-11} normalizing the x ray count to the measured stable isotope current. An example is shown in Fig. 1. The total detection efficiency, defined as the number of detected Ca $\mathrm{K}\alpha$ x rays per incident ^{41}Ca was determined from the standards to be 2.5%. The minimum detection limit for these standards was $\sim 2\times10^{-10}$, resulting from tails of the stable isotopes ^{40}Ca and ^{46}Ca which were not removed by the spectrometer. Use of a time-of-flight detector [10] should reject the ^{40}Ca component and a new ion source being planned for our accelerator, which includes an electrostatic analyzer, will significantly reduce these backgrounds. A further limitation in these measurements came from the low energy tails of the Ti and V $\mathrm{K}\alpha$ peaks (Fig. 1). The Ti x rays and the majority of the V x rays are caused by Ti ions passing through the spectrometer. Time-of-flight will allow rejection of this background. To further improve sensitivity for ^{41}Ca, by removing interferences from V x rays, we also made measurements in which the V foil was replaced by KCl powder, $2-5\,\mathrm{mg/cm^2}$, placed on adhesive tape. The Ca $\mathrm{K}\alpha$ detection efficiency for this target was ~1%. KCl could be an effective choice of target if improvements were made in the fabrication.

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Figure caption

Figure 1. PXAMS x ray spectra for a $^{41}Ca/^{40}Ca=10^{-9}$ standard. ^{41}Ca ions were incident on a V target, and the induced x rays were recorded using a high purity germanium detector. The majority of the V x rays are induced of incident Ti ions.

Table 1. Procedure for separation of calcium from urine samples as CaF_2

1. Precipitate calcium oxalate

Acidify urine sample to pH<1.9 with concentrated HCl.
Centrifuge 20 min at 1000g, and transfer 25 ml of the supernatant to 50-ml centrifuge tubes.
Add 25 ml saturated $(NH_4)_2C_2O_4$ at pH = 10 and 1.5 ml concentrated NH_4OH to the prepared urine.
Shake and fix the pH to 10 with concentrated NH_4OH.
Allow samples to sit 2.5 hours.
Centrifuge 20 min at 1000g, and discard supernatant.
Wash the precipitate with 2 ml 25 % $(NH_4)_2C_2O_4$.
Centrifuge 20 min at 1000g and discard supernatant (twice).
Wash the precipitate with 2 ml H_2O. Centrifuge 20 min. at 1000g and discard supernatant.
Dissolve the precipitate in 1 ml 4 M HNO_3; add H_2O to 50 ml.

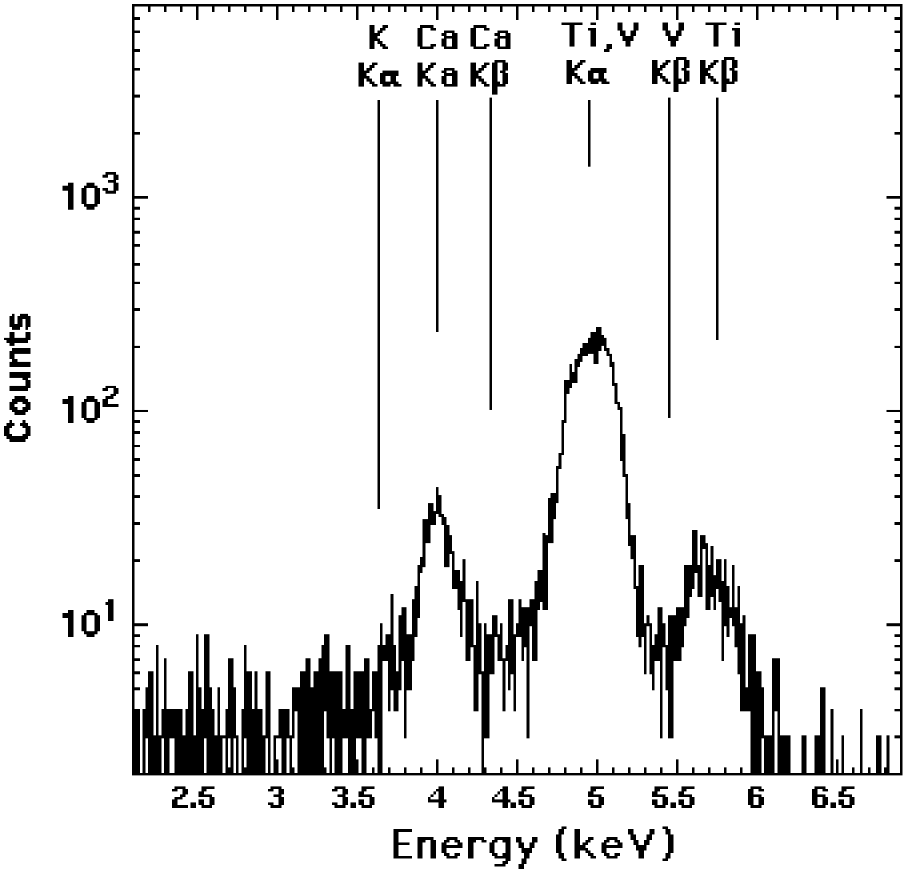
2. Separate calcium

Prepare small column with 1.5 ml resin.* Equilibrate the resin with 6 ml 0.08 M HNO_3.
Wash column with 4 ml of 4 M HNO_3 followed by 10 ml of H_20.
Load sample on the column.
Elute K and P etc. with 6 ml 0.08 M HNO_3.
Elute Ca with 4 ml 4 M HNO_3, and collect the eluate in a 15 ml centrifuge tube.
Dilute the eluate to 8.5 ml with H_2O.

3. Precipitate CaF_2

Add 6.5 ml HF (49%) to the diluted eluate. Shake and set overnight..
Centrifuge 20 min at 1000g and discard supernatant.
Transfer the precipitate to a microcentrifuge tube with 1 ml H_2O.
Centrifuge 10 min at 7000g and discard supernatant.
Wash with 0.75 ml H_2O. Centrifuge 10 min at 7000g and discard supernatant (twice).
Dry the sample in vacuum oven at 100°C for 20 hours.

*400 mesh resin, either Dowex 50W-X8 or Fisher Rexyn 101 (H).



K Kα
Ca Kα
Ca Kβ
Ti,V Kα
V Kβ
Ti Kβ
Counts
10^3
10^2
10^1
2.5
3
3.5
4
4.5
5
5.5
6
6.5
Energy (keV)

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